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Are birds stressed during long-term flights? A wind-tunnel study on circulating corticosterone in the red knot

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ABSTRACT

During endurance flight most birds do not feed and have to rely on their body reserves. Fat and protein is catabolised to meet the high energetic demands. Even though the hormonal regulation of migration is complex and not yet fully understood, the adrenocortical hormone corticosterone crystallizes to play a major role in controlling physiological traits in migratory birds during flight. However, results from field studies are partially equivocal, not least because data from birds during endurance flight are hard to get and present mostly a momentary shot. A wind-tunnel experiment offered the possibility to measure repeatedly under controlled conditions the effect of long flights on the stress hormone corticosterone. In a long-distance migrating shorebird, the red knot *Calidris canutus*, we measured plasma concentrations of corticosterone within 3 min and after a restraint time of 30 min directly after 2 h and 10 h non-stop flights, respectively, and during rest. Baseline corticosterone levels were unchanged directly after the flights, indicating that endurance flight did not affect corticosterone levels. The adrenocortical response to restraint showed the typical rise in birds during rest, while birds after a 2 or 10 h flight substantially decreased plasma corticosterone concentrations. We suggest that the negative adrenocortical response to restraint after flight is part of the mechanism to reduce the proteolytic effect of corticosterone to save muscle protein and to avoid muscle damaging effects.

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1. Introduction

During migration birds alternate between flight bouts and periods of staging for refuelling. Flight bouts in certain species, particularly shorebirds, can be long non-stop flights which may last in the most extreme cases for several days without food or water intake (Piersma and Baker, 2000; Battley et al., 2000; Gill et al., 2005, 2009; Piersma, 2007). To meet the high energy demands during flight, the birds have to rely exclusively on their body energy stores and metabolic water. After departure the metabolism changes from an anabolic, feeding phase to a catabolic phase during which the animal simultaneously deals with a long fast and a high energy expenditure (Rees et al., 1985; Jenni-Eiermann and Jenni, 1991). The birds change to a very high degree of fat catabolism within the first hour of flight (Jenni-Eiermann and Jenni, 1991; Schilch et al., 1996; Battley et al., 2000; Jenni-Eiermann et al., 2002).

The glucocorticoid corticosterone is thought to have an orchestrating role in the adjustment of the metabolism to the migratory period as well as for actual endurance flights. Experiments showed

that even slightly elevated circulating corticosterone promotes the availability of lipid energy substrate during energetically demanding states such as migration (Landys et al., 2004) and stimulates muscle protein breakdown and gluconeogenesis in order to maintain blood glucose (Kettelhut et al., 1988). All these mechanisms facilitate energy supply for endurance flight.

In field and aviary studies in shorebirds, plasma corticosterone levels have been found to increase concomitantly with the body mass of the migrants and reach a peak when birds initiate migratory flight (Piersma et al., 2000; Landys-Ciannelli et al., 2002). Elevated baseline levels of corticosterone have been observed in several species during the migratory period at rest and just after endurance flight, when the birds have landed (O'Reilly and Wingfield, 1995; Holberton et al., 1996; Landys-Ciannelli et al., 2002; Reneerkens et al., 2002). It is hypothesized that during a phase of increased energy demands hormone action maintains the metabolism within a heightened operating range, defined as state B by McEwen and Wingfield (2003). Whether this increase in baseline corticosterone concentration happens in anticipation to prepare the organism to state B (Landys-Ciannelli et al., 2002), or as a response to the increased demands, is not clear. In contrast to waders, low baseline levels of circulating corticosterone (<10 ng/ml) were found in several passerine species during migratory episodes

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(Schwabl et al., 1991; Gwinner et al., 1992; Jenni et al., 2000) suggesting a lack of activation of the hypothalamo–pituitary–adrenocortical axis (HPA). However, a direct comparison with non-migrating birds was only presented in one species, the garden warbler *Sylvia borin* (Schwabl et al., 1991). Corticosterone concentration of Garden warblers stopping-over in the Algerian Sahara were in the same (low) range as those of pre-migratory Garden warblers caught in Southwest Germany and Garden Warblers kept under laboratory conditions.

State B should not be confounded with a state of stress, an “emergency life-history stage”, during which in reaction to life-threatening stimuli corticosterone is released extensively to trigger physiological and behavioural changes which help the individual to survive (Wingfield et al., 1998). Such high stress levels of corticosterone were only observed in migrating birds with completely depleted fat stores and emaciated breast muscles (Gwinner et al., 1992; Jenni et al., 2000). In fact, for state B it was hypothesized that, analogous to inactive fasting birds (e.g. Cherel et al., 1988; Lynn et al., 2003), corticosterone levels increase when fat stores are depleted, in order to increase protein catabolism and to trigger a change in behaviour, i.e. a switch to searching for a stopover site and to landing (Jenni et al., 2000).

The adrenocortical response to acute stress, the ability to react to threatening events with a massive corticosterone release, may be modified during the migration period to cope with the particular behavioural and metabolic requirements of migration. The ability to react to a sudden stressor, reflected in the increase of corticosterone levels, is commonly measured with the handling and restraint stress protocol (Wingfield et al., 1994). Several studies suggest that migrating birds are indeed able to mount an adrenocortical stress response (Landys-Ciannelli et al., 2002; Tsipoura et al., 1999; O'Reilly and Wingfield, 1995; Schwabl et al., 1991), while others proposed elevated baseline corticosterone levels, and a reduced adrenocortical stress response in migratory birds compared with pre-migratory birds (Holberton, 1999; Holberton et al., 1996; Long and Holberton, 2004). According to the Migration Modulation Hypothesis (Holberton et al., 1996) elevated baseline corticosterone levels facilitate migratory fattening during the refuelling phase (but see Piersma et al., 2000), while the adrenocortical response to acute stress should be reduced to prevent an even higher level of plasma corticosterone, and thus causing breakdown of skeletal muscles.

In summary, it remains unclear whether baseline corticosterone is indeed increased as a preparation for, or a response to, endurance flight. It also remains unclear whether the adrenocortical stress response is dampened during the migratory phase. The conflicting results could be due to the fact that all studies to date could not sample the same individual during flight as well as before or after flight. Moreover, in most field studies the history of a bird is unknown. The observed plasma corticosterone concentrations could be evoked by environmental influences such as adverse weather conditions or by a different physiological state of the individual (fasting, exercising, feeding). Wind-tunnel studies offer the unique possibility for controlled flight experiments (Pennycuik et al., 1997), which allow to take into account factors such as length of the flight and environmental conditions. In this study we measured baseline plasma corticosterone levels and the adrenocortical response to restraint in red knots during the autumn migration period before they had any possibility to fly, during mid-flight in a wind-tunnel and one day after several long flights. The design enabled us to investigate whether baseline plasma corticosterone levels and the adrenocortical response to restraint were affected by actual flight (2 or 10 h) or a period of flight experience in comparison with resting, untrained birds.

2. Materials and methods

2.1. Birds and experimental design

In this study we used data from eight red knots which successfully flew in the Lund wind-tunnel. They were part of 23 juvenile red knots trapped at the night-time roost site Richel in the western Dutch Wadden Sea (53°16'N, 5°08'E) on 29 and 30 August 2000. They were kept at the NIOZ experimental shorebird facility on Texel, The Netherlands (53°05'N, 4°75'E) and transported to the wind-tunnel in Lund, Sweden in two batches in the following autumn. When batch 1 (10 birds) was in Lund for the experiments, the other group (batch 2, 10 birds) remained in the Netherlands. Hence, batch 2 had a longer period between trapping and experiment than batch 1, and we therefore entered batch as variable in the statistical analyses. However, for both groups experiments were conducted during the species migration period (batch 1: 11–30 Sept; batch 2: 27 Oct–16 Nov), the birds accumulated high fat levels characteristic of knots in migratory state, and they flew voluntarily for long sessions (2–10 h) in the wind-tunnel (Hasselquist et al., 2007). For details about housing and assigning birds to either a group flown in the wind-tunnel or a control group not flown in the wind-tunnel, see Hasselquist et al. (2007). Since the birds were also tested for the effect of long flights on immune response, they were injected with a diphtheria-tetanus vaccine two times, the first time one month before flight treatment and the second time just before flight treatment started (for details see Hasselquist et al., 2007). Details about the Lund wind-tunnel and the flight performance of red knots can be found in Pennycuik et al. (1997), Lindström et al. (2000), Kvist et al. (2001) and Jenni-Eiermann et al. (2002). Briefly, one or two birds flew in an open test section that is 1.2 m wide, 1 m high and 2 m long, with transparent walls and a 50 cm wide opening that allows a person to work with the birds in the air current. During the flight treatment wind speed was set at 15 ms⁻¹ and temperature to +10–+14 °C, which is below the critical temperature avoiding dehydration in flying knots (Kvist et al., 2001).

Experiments were done during 1 week. Out of 12 birds trained in the wind-tunnel, six successfully completed a flight program of a total of 26–28.75 h within this week. This is equivalent to 1400–1550 km of flight in still air. The successful flyers flew on three to five of the 7 days. All individuals flew two separate 10 h sessions (then always with a non-flying day in between), and in addition a varying number of 1, 2 and 4 h flights. There was only one flight per day. The birds were flown either alone or in pairs. Paired flights were initiated to maximize the total flight time achieved within the week. On four occasions we had paired flights lasting 10 h non-stop. Blood for “in-flight” cort measurements was sampled on a sub-set of these flights (flights of 2 or 10 h). Two additional birds did not complete the entire flight program, but flew successfully one or more 2 or 10 h flights in which blood was sampled. These additional flights were included in the study of in-flight cort measurements. In the seven cases when birds completed a 2 h flight they lost on average 5.8 g (pre flight: 134.6 ± 8.7; post flight: 128.8 ± 8.9) and in the five cases when birds completed a 10 h flight they lost on average 9.8 g (pre-flight: 139.5 ± 9.91; post-flight: 123.69 ± 9.7 g). Between the flights the birds were kept in the aviary together with their flock mates.

2.2. Blood sampling

The brachial vein was punctured and a total of 75–125 µl of blood collected in heparinized microcapillary tubes. All birds were sampled within 3 min after capture. The birds were then kept in a small darkened cage and a second sample was taken ca 30 min after catching (the restraint response sample). Blood samples taken

within 3 min were assumed to represent baseline levels. Blood samples were immediately centrifuged for seven min at 3000 rpm and stored at -30°C within an hour. The samples were sent frozen to Switzerland on 14 May 2002 where they were analysed for corticosterone.

A blood sample (within 3 min and after 30 min) was taken 1 day after the birds arrived in Lund (pre-experimental), immediately after they had completed a 2 h- or a 10 h-flight (in-flight) and one day after the experimental period had finished (post-experimental). For in-flight samples, there were four individuals with a 2 h- and 10 h-flight sample, three individuals with only a 2 h-flight sample, and one individual with only a 10 h-flight sample. Hence, there are 7 2 h-flights samples and 5 10 h-flights samples.

2.3. Corticosterone analysis

Plasma corticosterone concentration was determined using an enzyme immuno assay (Munro and Lasley, 1988). Corticosterone in 10 μl of plasma (diluted 1:20 in $\text{H}_2\text{O}_{\text{bidest}}$) was extracted with 4 ml dichloromethane, re-dissolved in phosphate buffer and analysed in triplicates. A sheep-anti-corticosterone polyclonal antibody was used in a final dilution of 1:8000 (Chemicon Int.). The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. A plasma pool from chicken *Gallus domesticus* was used as internal control on each plate. The intra-assay coefficient of variation was 7.2% and the inter-assay coefficient of variation 18.9%. The assays were performed in duplicate in June 2002.

2.4. Statistics

Data were analysed with Mixed Model Analysis (residual maximum-likelihood analysis, REML; Patterson and Thompson, 1971) in Genstat, release 6.1. This procedure is appropriate for the analysis of repeated measurements from the same individuals in an unbalanced design (i.e. unequal numbers of data from each individual).

The effect of flight and restraint on corticosterone concentration (dependent variable) was tested by including the variables experimental condition (pre-experimental, 2 h-flight, 10 h-flight and post-experimental), handling and restraint time (3 and 30 min), body mass, batch (1 and 2) and the interaction terms as fixed effects and the individuals as random effects in a Mixed Model. Time of day was tested in a first model but since there was no significant effect ($P = 0.733$) we excluded this variable.

3. Results

To assess baseline corticosterone and the adrenocortical response to restraint during endurance flight activity, we compared in-flight corticosterone levels after 2 h- and 10 h-flights with the pre- and post-experimental values, the latter taken one day after the last flight (Table 1). Baseline corticosterone in-flight levels after a 2 h- or a 10 h-flight were not significantly different from values before and after the flight sessions ($P = 0.078$; Fig. 1). The 2 h-flight samples had slightly higher baseline corticosterone levels than the pre-experimental samples, but the difference was not significant (post-hoc test: paired-sample t -test, $N = 7$, $t = -1.94$, $P = 0.10$).

The corticosterone levels after 30 min of restraint were markedly decreased after a 2 h- or a 10 h-flight (Fig. 1), while there was the usual increase in corticosterone in resting birds (pre-experimental and post-experimental; interaction term restraint time \times treatment, $P < 0.001$; Table 1). There was no significant effect of body mass or batch on corticosterone levels. Post-hoc, a paired-sample t -test analysis confirmed that values 30 min after flight were significantly lower than baseline values when all flights

Table 1

Effect of treatment (pre-experimental, 2 h-flight, 10 h-flight, post-experimental), restraint time (within 3 min or after 30 min after capture), body mass, batch and the interaction term restraint time \times treatment on circulating corticosterone levels in red knots, tested in a REML-analysis (deviance: 272.92, d.f. = 44; random effects were individuals). The effects \pm SE, degrees of freedom d.f. and Wald statistics are given with their significance levels. The non-significant interaction terms were omitted. For treatment, effect sizes are given relative to pre-experimental (set at 0), for restraint time relative to baseline level (set at 0), and for batch relative to batch 1 (set at 0).

Fixed effects	Effects \pm SE	d.f.	Wald statistics/d.f.	P
Treatment				
2 h-flight	11.74 \pm 4.67	3	2.27	0.078
10 h-flight	4.62 \pm 4.67			
Post-experimental	-1.12 \pm 4.67			
Restraint time, 30 min	7.53 \pm 4.13	1	2.36	0.123
Body mass	0.15 \pm 0.13	1	0.60	0.437
Batch, number 2	-5.53 \pm 4.05	1	1.86	0.172
Restraint time \times Treatment		3	7.35	<0.001
30 min \times 2 h-flight	-24.01 \pm 6.24			
30 min \times 10 h-flight	-20.86 \pm 6.24			
30 min \times post-exp	-4.21 \pm 6.24			
Constant	17.57 \pm 3.82			

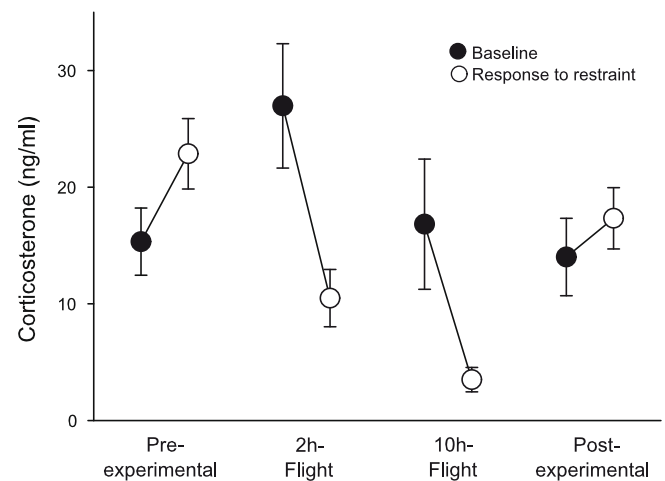


Fig. 1. Plasma corticosterone concentrations (mean \pm S.E.) within 3 min (baseline) and after 30 min of restraint (restraint response) of eight red knots measured pre-experimental, after 2 h flights, after 10 h flights and post-experimental.

were combined (2 h- and 10 h-flights: $N = 12$, $t = 3.73$, $P = 0.003$). This was also the case for the 2 h-flights ($N = 7$, $t = 2.79$, $P = 0.032$), but not for the 10 h-flights ($N = 5$, $t = 2.28$, $P = 0.085$), possibly because of lack of statistical power. Pre- and post-experimentally corticosterone levels increased significantly after 30 min of restraint (pre- and post-experimental combined, $N = 16$, $t = -2.47$, $P = 0.026$, only pre-experimental birds: $N = 8$, $t = -1.96$, $P = 0.09$; only post-experimental birds: $N = 8$, $t = -1.54$, $P = 0.17$). This agreed with a highly significant adrenocortical response to restraint in control birds not-flown (data not shown).

4. Discussion

4.1. Corticosterone levels during actual flight

This is the first study investigating circulating corticosterone in birds after flights of known duration and controlled conditions. In-flight baseline corticosterone concentrations of red knots were not significantly different after 2 h- and 10 h-flights, respectively, to those before or after the experimental flight period. However, the trend to increased corticosterone levels after 2 h-flights suggests that we might have missed a transient increase of corticosterone during the first 1–2 h after the onset of flight when the metabolism

of red knots changes to maximum lipid catabolism (Jenni-Eiermann et al., 2002). The pre- and post-experimental baseline levels of the birds flown in the wind-tunnel did not differ from control birds not flown in the wind-tunnel, but kept under the same conditions (Hasselquist et al., 2007). The average baseline corticosterone concentration of the red knots in our study (15.7 before and 14.00 ng/ml after the experimental flights, see Fig. 1) is within the range reported in other studies on the same species (10–30 ng/ml; Piersma et al., 2000; Piersma and Ramenofsky, 1998; Reneerkens et al., 2002). Hence, it seems very unlikely, that the baseline levels were affected by the immune challenge the knots experienced before the flight experiments (Hasselquist et al., 2007).

The only other corticosterone data from experimentally controlled physical activity in birds came from domestic birds. Homing pigeons *Columbia livia* after flights of more than 180 km (Haase et al., 1986), as well as pigeons and ducks exercising in a treadmill, showed slightly elevated corticosterone concentrations, well below the levels after stimulation with the adrenocorticotrophic hormone (ACTH) (Rees and Harvey, 1987) or handling (Harvey and Phillips, 1982). These results would – in contrast to our 10 h flights – indicate a small stimulation of the adrenocortical axis due to exercise. One might also hypothesize that domestic animals have a different regulation of the HPA-axis than wild, migrating birds. Behavioural adaptations including hyperphagia to build up large amounts of fat (Bairlein, 1985) as well as physiological adaptations to maximize the use of lipids as fuel were only shown in free-living migrants (Guglielmo et al., 1998; Jenni and Jenni-Eiermann, 1998), suggesting a differentially regulated metabolism during migratory flight. Passerine migrants captured directly out of nocturnal migratory flight showed low corticosterone levels (<10 ng/ml; Gwinner et al., 1992; Schwabl et al., 1991), in the same range as baseline levels reported for three of the same species in other life-history stages (Schwabl et al., 1991: garden warbler <10 ng/ml; Silverin et al., 1997: willow warbler *Phylloscopus trochilus*, 4–20 ng/ml; Silverin and Wingfield, 1998: pied flycatcher *Ficedula hypoleuca*, 5–15 ng/ml). These studies also suggest that, just as in homing pigeons (Haase et al., 1986), any increase in corticosterone release due to exercise would be small.

In contrast, field studies analyzing blood samples from shorebirds (bar-tailed godwits, Landys-Ciannelli et al., 2002; red knots, Reneerkens et al., 2002) just after long northward flights showed elevated corticosterone levels, but with large interindividual variation (mean: 18 ng/ml, range: about 2–64 ng/ml for bar-tailed godwits; mean: 58 ng/ml, range: 22–101 ng/ml for red knots). In free-living passerines caught during or directly after migratory flights high levels of corticosterone (about 40 ng/ml) were only found in individuals with emaciated flight muscles and no energy stores left, while in all other migrant passerines sampled, corticosterone concentrations were well below 20 ng/ml (Jenni et al., 2000). The large variation in corticosterone levels of newly arrived bar-tailed godwits and red knots suggests that corticosterone is not a primary regulator of behaviours during migration. Instead, corticosterone seems to be released in response to certain circumstances, such as starvation, health problems, extreme ambient temperatures (Jeronen et al., 1976) or unfavourable weather conditions. The sample of shorebirds and passerines found with high circulating corticosterone levels during and after natural migration flights may well have comprised the most-challenged individuals, birds that were most prone to land or to fly low over ground.

4.2. Effect of actual flight on the adrenocortical response to restraint

We demonstrated that the decrease in plasma corticosterone levels was a genuine response to half an hour of restraint after flight, not influenced by body mass or flight duration or any inter-

action term (Table 1). As our keeping of red knots in a small cage for half an hour before blood sampling differs from the handling and stress protocol according to Wingfield et al. (1998), it might be argued that our treatment might not cogently evoke stress. However, in our experiment the red knots in the resting group consistently showed an increase of plasma corticosterone after the same treatment, indicating that our restraint treatment produced the same effect as the protocol developed by Wingfield et al. (1998). The strong response to restraint in the resting birds also shows, that the immune challenge the birds had experienced, does not suppress the HPA axis. We know of no other study with exercising vertebrates demonstrating a decrease of plasma corticosterone with restraint in all individuals examined. Nevertheless, this novel pattern may be adaptive.

Migratory endurance flight can be regarded as fasting with a high energy expenditure (Jenni and Jenni-Eiermann, 1998; Battley et al., 2001). Birds derive their energy not only from body fat stores, but also from proteinous tissue (Jenni and Jenni-Eiermann, 1998). In red knots flying in the wind-tunnel, plasma uric acid levels were increased after 1 h of flight, indicating a catabolism of proteinous tissues (Jenni-Eiermann et al., 2002). Protein catabolism and gluconeogenesis from amino acids are promoted by corticosterone and enable to maintain glucose and the level of intermediates of the citric acid cycle which are constantly drawn away by the anaerobic flux (Kettelhut et al., 1988; Jenni and Jenni-Eiermann, 1998). As soon as a bird interrupts its flight, about 8–10 times less energy is needed and fat and protein catabolism must be reduced. In several species of free-flying passerines caught out of their autumn migratory flight, the metabolic changes indicated a quick response to the reduced energetic demands during the first 20 min of recovery and subsequently a postexercise ketosis and a reduction of lipolysis and proteolysis (Jenni-Eiermann and Jenni, 2001). Therefore, a reduction in circulating corticosterone probably helps to reduce protein catabolism after flight and thus saves muscle tissue together with the observed postexercise ketosis. This is of great importance for a migratory bird, which has to maintain flight capability for foraging, escape flights and the continuation of migration (van den Hout et al., 2006; Dietz et al., 2007).

Alternatively, a reduction of corticosterone may reflect a psychological reward. A study on rats showed that animals which received an expected reward, decreased corticosterone and ACTH by about 20–32% within 15 min, while rats which were frustrated showed an increase of corticosterone and ACTH (Romero et al., 1995). If the red knots learned that restraint indicated the end of a demanding session of wind-tunnel flight, even though flight in the wind-tunnel was voluntary, the restraint might decrease the activity of the HPA-axis. However, if this explanation would be true, it would remain unclear which metabolic advantage the birds should have by decreasing corticosterone concentration below baseline levels 30 min after flight. Moreover, the birds were free to stop flying at any time, as some birds demonstrated which refused to fly or did not complete their flight program (see also Hasselquist et al., 2007).

5. Conclusions

This study showed that corticosterone was if anything only slightly increased after 2 h and not increased at all after 10 h of actual flight. Hence, long flights in migrating birds with sufficient energy stores do not necessitate up-regulation of corticosterone to a stage of increased energetic demands as would follow from the model developed by McEwen and Wingfield (2003). However, it might still be possible that a slight or transitory elevation of corticosterone as observed in homing pigeons might have been missed. The sharp reduction of corticosterone 30 min after flight despite restraint suggests that corticosterone is involved in the down-reg-

ulation of lipolysis and proteolysis. We propose that the negative adrenocortical response to restraint may be part of a mechanism to reduce proteolysis after the flight is accomplished.

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